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(54) Title: NUCLEASE RESISTANT, PYRIMIDINE MODIFIED OLIGONUCLEOTIDES THAT DETECT AND MODULATE GENE EXPRESSION

(57) Abstract

Oligonucleotide analogs are provided having improved nuclease resistance. Modifications of selected nucleotides through substitutions on the pyrimidine ring are disclosed. Certain preferred embodiments comprise the inclusion of said modified nucleotides at a plurality of sites, especially at the 3' end of a selected oligonucleotide analog.

NUCLEASE RESISTANT, PYRIMIDINE MODIFIED OLIGONUCLEOTIDES THAT DETECT AND MODULATE GENE EXPRESSION

FIELD OF THE INVENTION

This invention relates to the design, synthesis
5   and application of nuclease resistant oligonucleotides that
are useful for antisense oligonucleotide therapeutics,
diagnostics, and research reagents. Pyrimidine modified
oligonucleotides that are resistant to nuclease degradation
and are capable of modulating the activity of DNA and RNA
10 are provided. Methods for modulating the production of
proteins utilizing the modified oligonucleotides of the
invention are also provided.

BACKGROUND OF THE INVENTION

It is well known that most of the bodily states
15 in mammals including infectious disease states, are
effected by proteins. Such proteins, either acting
directly or through their enzymatic functions, contribute
in major proportion to many diseases in animals and man.
Classical therapeutics has generally focused upon
20 interactions with such proteins in efforts to moderate
their disease causing or disease potentiating functions.
Recently, however, attempts have been made to moderate the
actual production of such proteins by interactions with
molecules that direct their synthesis, intracellular RNA.
25 By interfering with the production of proteins, it has been
hoped to effect therapeutic results with maximum effect and

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minimal side effects. One approach for inhibiting specific
gene expression is the use of oligonucleotides and
oligonucleotide analogs as antisense agents.

Antisense methodology is the complementary
5   hybridization of relatively short oligonucleotides to
single-stranded mRNA or single-stranded DNA such that the
normal, essential functions of these intracellular nucleic
acids are disrupted. Hybridization is the sequence
specific hydrogen bonding of oligonucleotides to Watson-
10 Crick base pairs of RNA or single stranded DNA. Such base
pairs are said to be complementary to one another.

Naturally occurring event that lead to disruption
of the nucleic acid functions are discussed by Cohen in
Oligonucleotides: Antisense Inhibitors of Gene Expression,
15 (CRC Press, Inc., Boca Raton FL, 1989). These authors
proposes two possible types of terminating events. The
first, hybridization arrest, denotes a terminating event in
which the oligonucleotide inhibitor binds to the target
nucleic acid and thus prevents, by simple steric hindrance,
20 the binding of essential proteins, most often ribosomes, to
the nucleic acid. Methyl phosphonate oligonucleotides; P.
S. Miller & P.O.P. Ts'O, *Anti-Cancer Drug Design*," Vol. 2,
pp. 117-128 (1987); and α-anomer oligonucleotides, Cohen
J.S. ed., *Oligonucleotides: Antisense Inhibitors of Gene*
25 *Expression*, (CRC Press, Inc., Boca Raton FL 1989), are two
of the most extensively studied antisense agents that are
thought to disrupt nucleic acid function by hybridization
arrest.

A second type of terminating event for antisense
30 oligonucleotides involves enzymatic cleavage of the
targeted RNA by intracellular RNase H. The oligonucleotide
or oligonucleotide analog, which must be of the deoxyribo
type, hybridizes with the targeted RNA and this duplex
activates the RNase H enzyme to cleave the RNA strand, thus
35 destroying the normal function of the RNA.
Phosphorothioate oligonucleotides are a prominent example

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of an antisense agent that operates by this type of
antisense terminating event.

Considerable research is being directed to the
application of oligonucleotides and oligonucleotide analogs
5   as antisense agents for therapeutic purposes. Applications
of oligonucleotides as diagnostics, research reagents, and
potential therapeutic agents require that the
oligonucleotides or oligonucleotide analogs be synthesized
in large quantities, be transported across cell membranes
10 or taken up by cells, appropriately hybridize to targeted
RNA or DNA, and subsequently terminate or disrupt nucleic
acid function. These critical functions depend on the
initial stability of oligonucleotides towards nuclease
degradation.

15   A serious deficiency of naturally occurring
oligonucleotides and existing oligonucleotide analogs for
these purposes, particularly those for antisense
therapeutics, is the enzymatic degradation of the
administered oligonucleotide by a variety of ubiquitous
20 nucleolytic enzymes, intracellularly and extracellularly
located, hereinafter referred to as "nucleases". It is
unlikely that unmodified oligonucleotides will be useful
therapeutic agents because they are rapidly degraded by
nucleases. Modification of oligonucleotides to render them
25 resistant to nucleases is therefore greatly desired.

Modifications of oligonucleotides to enhance
nuclease-resistance have heretofore generally taken place
on the sugar-phosphate backbone, particularly on the
phosphorus atom. Phosphorothioates, methyl phosphonates,
30 phosphoramidates, and phosphotriesters (phosphate
methylated DNA) have been reported to have various levels
of resistance to nucleases. However, while the ability of
an antisense oligonucleotide to bind to specific DNA or RNA
with fidelity is fundamental to antisense methodology,
35 modified phosphorus oligonucleotides, while providing
various degrees of nuclease resistance, have generally
suffered from inferior hybridization properties.

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One reason for this inferior hybridization may be due to the prochiral nature of the phosphorus atom. The modifications on the internal phosphorus atoms of modified phosphorous oligonucleotides result in Rp and Sp stereo-

5   isomers. Since a practical synthesis of stereoregular oligonucleotides (all Rp or Sp phosphate linkages) is unknown, oligonucleotides with modified phosphorus atoms have n^2 isomers with n equal to the length or the number of the bases in the oligonucleotide. Furthermore,

10 modifications on the phosphorus atom have unnatural bulk about the phosphodiester linkage that interferes with the conformation of the sugar-phosphate backbone and consequently the stability of the duplex. The effects of phosphorus atom modifications cause inferior hybridization

15 to the targeted nucleic acids relative to the unmodified oligonucleotide hybridizing to the same target.

The relative ability of an oligonucleotide to bind to complementary nucleic acids may be compared by determining the melting temperature of a particular

20 hybridization complex. The melting temperature (T_m), a characteristic physical property of double helixes, denotes the temperature in degrees centigrade at which 50% helical versus coil (unhybridized) forms are present. T_m is measured by using the UV spectrum to determine the

25 formation and breakdown (melting) of hybridization. Base stacking that occurs during hybridization, is accompanied by a reduction in UV absorption (hypochromicity). Consequently, a reduction in UV absorption indicates a higher T_m. The higher the T_m, the greater the strength of

30 the binding of the strands. Non-Watson-Crick base pairing has a strong destabilizing effect on the T_m. Consequently, absolute fidelity of base pairing is likely necessary to have optimal binding of an antisense oligonucleotide to its targeted RNA.

35 Considerable reduction in the hybridization properties of methyl phosphonates and phosphorothioates has been reported: see Cohen, J.S., ed. *Oligonucleotides:*

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Antisense Inhibitors of Gene Expression, (CRC Press, Inc.,
Boca Raton FL, 1989). Methyl phosphonates have a further
disadvantage in that the duplex it forms with RNA does not
activate degradation by RNase H as an terminating event,
but instead acts by hybridization arrest that can be
reversed due to a helical melting activity located on the
ribosome. Phosphorothioates are highly resistent to most
nucleases. However, phosphorothioates typically exhibit
non-antisense modes of action, particularly the inhibition
of various enzyme functions due to nonspecific binding.
Enzyme inhibition by sequence-specific oligonucleotides
undermines the very basis of antisense chemotherapy.

      Therefore, oligonucleotides modified to exhibit
resistance to nucleases, to activate the RNase H
terminating event, and to hybridize with appropriate
strength and fidelity to its targeted RNA (or DNA) are
greatly desired for anti-sense oligonucleotide diagnostics,
therapeutics and research.

OBJECTS OF THE INVENTION

      It is a principal object of the invention to
provide oligonucleotide analogs for use in antisense
oligonucleotide diagnostics, research reagents, and
therapeutics.

      It is further an object of the invention to
provide such oligonucleotide analogs that are effective in
modulating the activity of a DNA or an RNA.

      Another object of the invention is to provide
such oligonucleotide analogs that are less likely to invoke
undesired or toxic side reactions.

      Yet another object of the invention is to provide
research and diagnostic methods and materials for assaying
bodily states in animals, especially diseased states.

      A further object of the invention is to provide
therapeutic and research methods and materials for the
treatment of diseases through modulation of the activity of
DNA and RNA.

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These and other objects will become apparent to persons of ordinary skill in the art from a review of the present specification and attendant claims.

SUMMARY OF THE INVENTION

5   In accordance with the present invention, compositions that are resistant to nuclease degradation and that modulate the activity of DNA or RNA are provided. These compositions are comprised of oligonucleotides that incorporate modified pyrimidine bases therein, the

10   targeting portions of which are specifically hybridizable with preselected nucleotide sequences of single stranded or double-stranded DNA and RNA. The pyrimidine modified oligonucleotides recognize and form double strands with single stranded DNA and RNA or triple strands with double

15   stranded DNA and RNA.

The nuclease resistant oligonucleotides consist of a strand of nucleic acid bases linked together through linking groups. The target portion of the nuclease resistant oligonucleotides may range in length from about 5

20   to about 50 nucleic acid bases. However, in accordance with preferred embodiments of this invention, a target sequence 15 bases in length is believed to be optimal.

The pyrimidine modified oligonucleotides of the invention incorporate bases including pyrimidines such as

25   thymine, uracil or cytosine, or purines such as guanine or adenine, or modifications thereof, arranged in a selected sequence. The sugar moieties of such bases may be either deoxyribose or ribose sugars. The groups linking the bases together may be the usual sugar phosphate nucleic acid

30   backbone, but may also be modified as a phosphorothioate, alkylphosphonates such as methylphosphonate, phosphotriester, phosphoamidate or other backbone modifications useful to further enhance the pyrimidine modified oligonucleotide properties. For example, the

35   phosphodiester linkage may also be replaced by a carbon or ether linkage.

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In accordance with other preferred embodiments of
this invention, the targeting portion may be an analog of
an oligonucleotide wherein at least one of the pyrimidine
bases, thymine, cytosine, or uracil, have been substituted
5   with a modified nucleic acid base, which is faithful to the
Watson-Crick hydrogen-bonding base pair rules (T to A, C to
G and U to A), yet confers nuclease resistance to the
oligonucleotide. Modification of the pyrimidine base or
bases preferably occurs at the 5 or 6 positions of the
10 pyrimidine ring. In the alternative, one or more of the
pyrimidine bases may be modified at both the 5 and 6
positions of the pyrimidine ring.

The substitutions that may occur at the 5 and 6
positions may include additions of hetero atoms for the
15 carbon atoms at the 5 and 6 positions of the pyrimidine or
the addition methyl, hydroxyl, ether, alcohol, benzyl, or
phenyl groups. The modifications may also involve the
addition of nitro groups, thiol groups, halogen groups or
halocarbon groups including fluorocarbon groups. In the
20 alternative, the modification may be a carbocyclic or
heterocyclic ring that is formed via fusion of a substrate
to the 5 and 6 positions of the pyrimidine ring. The
additions that occur at the 5 and 6 positions may, but need
not be, the same.

25 The resulting novel oligonucleotides are
resistant to nuclease degradation and exhibit
hybridization properties of higher quality relative to wild
type (DNA-DNA and RNA-DNA) duplexes and the presently known
phosphorus modified oligonucleotide antisense duplexes
30 containing phosphorothioates, methylphosphonates,
phosphoramidates and phosphotriesters. The invention is
also directed to methods for modulating the production of a
protein by an organism comprising contacting the organism
with a composition formulated in accordance with the
35 foregoing considerations. It is preferred that the RNA or
DNA portion that is to be modulated be preselected to
comprise that portion of DNA or RNA that codes for the

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protein whose formation is to be modulated. The targeting
portion of the composition to be employed is, thus,
selected to be complementary to the preselected portion of
DNA or RNA, that is to be an antisense oligonucleotide for
5   that portion.
        This invention is also directed to methods for
treating an organism having a disease characterized by the
undesired production of a protein. This method comprises
contacting the organism with a composition in accordance
10 with the foregoing considerations. The composition is
preferably one which is designed to specifically bind with
messenger RNA that codes for the protein whose production
is to be inhibited.
        The invention further is directed to diagnostic
15 methods for detecting the presence or absence of abnormal
RNA molecules or abnormal or inappropriate expression of
normal RNA molecules in organisms or cells.
        This invention is also directed to methods for
the selective binding of RNA for research and diagnostic
20 purposes. Such selective, strong binding is accomplished
by interacting such RNA or DNA with compositions of the
invention that are resistance to degradative nucleases and
that hybridize stronger and with greater fidelity than
known oligonucleotides or oligonucleotide analogs.
25 **DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS**
        The compositions useful for modulating the
activity of an RNA or DNA molecule in accordance with this
invention generally comprise a modified pyrimidine
containing oligonucleotide of a target sequence that is
30 specifically hybridizable with a preselected nucleotide
sequence of single stranded or double stranded DNA or RNA
molecule and that is nuclease resistant.
        It is generally desirable to select a sequence of
DNA or RNA for or which is involved in the production of
35 proteins whose synthesis is ultimately to be modulated or
inhibited in entirety. The targeting portion of the
composition is generally an oligonucleotide analog. It is

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synthesized, conveniently through solid state synthesis of
known methodology, to be complementary to or at least to be
specifically hybridizable with the preselected nucleotide
sequence of the RNA or DNA. Nucleic acid synthesizers are
5   commercially available and their use is generally
understood by persons of ordinary skill in the art as being
effective in generating nearly any oligonucleotide of
reasonable length that may be desired.

In the context of this invention, the term
10 "oligonucleotide" refers to a plurality of joined
nucleotide units formed in a specific sequence from
naturally occurring bases and pentofuranosyl sugar groups
joined through an internucleotide linking group by native
phosphodiester bonds. These nucleotide units may be
15 nucleic acid bases such as guanine, adenine, cytosine,
thymine or uracil. The sugar group may be a deoxyribose or
ribose sugar. This term refers to both naturally occurring
or synthetic species formed from naturally occurring
subunits.

20   "Oligonucleotide analog" as the term is used in
connection with this invention, refers to moieties that
function similarly to oligonucleotides but which have non-
naturally occurring portions. Oligonucleotide analogs may
have altered sugar moieties or inter-sugar linkages, for
25 example, phosphorothioates and other sulfur containing
species which are known for use in the art.
Oligonucleotide analogs may also comprise altered base
units or other modifications consistent with the spirit of
this invention, and in particular such modifications as may
30 increase nuclease resistance of the oligonucleotide
composition in order to facilitate antisense therapeutic,
diagnostic or research reagent use of a particular
oligonucleotide.

It is generally preferred for use in some
35 embodiments of this invention that some positions of the
nucleotide base be substituted in order to increase the
nuclease resistance of the composition while maintaining

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the integrity of the oligonucleotide binding capabilities.
Such substitutions may occur at the 5 or 6 position of one
or more pyrimidine rings by substituting a heteroatom for a
carbon atom of the pyrimidine ring at these positions. In

5   the alternative, the nuclease resistance of the
oligonucleotide may be increased by addition of a
substituent group at the 5 and 6 positions of the
pyrimidine ring.

    Substituent groups may be methyl, hydroxyl,
10   alkoxy, alcohol, ester, benzyl, phenyl, nitro, thiol,
thioalkoxy, halogen, halocarbon or fused carbon rings or
heteroatom containing rings. In accordance with some
preferred embodiments of the invention, substitutions of
the pyrimidine ring may be aza at the 5 or 6 or both the 5
15   and 6 position. In accordance with other preferred
embodiments of the invention, substituent groups added to
the 5 or 6 positions may be one or more of nitro-,
methyl-, bromo-, iodo-, chloro-, fluoro-, trifluoro-,
trifluoromethyl-, 2,4- dinitrophenyl-, mercapto-, or
20   methylmercapto- groups. Other additions that may also be
preferred for use against nuclease degradation are ethers,
thioethers, alcohols and thioalcohols such as HS-C-, MeS-
C-, OH-C-, MeO-C-, HOCH$_2$-C-, and cyclopentyl, cyclohexyl and
imidazo rings fused to the pyrimidine ring via the 5 and 6
25   positions of the pyrimidine ring. Accordingly, some
preferred embodiments of this invention may incorporate a
modified pyrimidine base or bases having the structure:
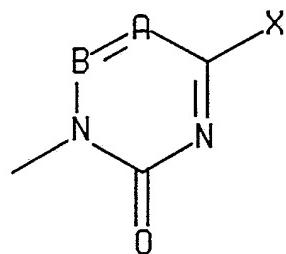
wherein   X= OH or NH$_2$, and A and B may be the same or
different and may be: C-lower alkyl, N, C-CF$_3$, C-F, C-Cl,

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C-Br, C-I, C-halocarbon including C-fluorocarbon, $C-NO_2$, C-
OCF_3, C-SH, $C-SCH_3$, C-OH, C-O-lower alkyl, $C-CH_2OH$, $C-CH_2SH$,
$C-CH_2SCH_3$, $C-CH_2OCH_3$, $C-NH_2$, $C-CH_2NH_2$, $C-alkyl-NH_2$, C-benzyl,

C-aryl, C-substituted aryl, C-substituted benzyl; or one of
5   A and B are as above and the other is C-H; or together A
and B are part of a carbocyclic or heterocyclic ring fused
to the pyrimidine ring through A and B. It is preferred
that one or both of A and B be C-lower alkyl, C-O-lower
alkyl, C-OH, C-phenyl, C-benzyl, C-nitro, C-thiol, C-
10   halocarbon, or C-halogen. In accordance with other
preferred embodiments, at least one of A and B is C-halogen
or C-halocarbon including C-fluorocarbon, especially C-
trifluoromethyl. Other fluorocarbon include $C-C(CF_3)_3$, C-
CF_2-CF_3 and $C-CF_2-CF_2-CF_3$. Halogen includes fluorine,
15   bromine, chlorine and iodine.

        In accordance with other preferred embodiments,
one or both of A and B are nitrogen atoms. It is still
more preferred that A be nitrogen. In other embodiments, A
is $C-CH_3$ or $C-CF_3$ and B is nitrogen or A is C-Br and B is
20   nitrogen.

        It is preferred that at least one modified
pyrimidine be at one end of said oligonucleotide analog,
especially at the 3' end of said oligonucleotide analog.
In accordance with other, preferred embodiments, the
25   oligonucleotide analogs contain up to about 3 modified
pyrimidines incorporated at the 3' end of said
oligonucleotide. In other embodiments, at least about 1%
of said pyrimidines are modified. It is preferred in
accordance with other embodiments to have greater
30   quantities of modifications, such as at least about 10% of
said pyrimidines, or even, 25%, 50% or substantially all of
said pyrimidines.

        The sugar moieties attached to the modified
pyrimidine can be either a ribose or deoxyribose sugar.
35   The sugar linking groups of the modified pyrimidine can be
modified to a phosphorothioate, alkyl phosphonate including
methyl phosphonate, phosphoamidate, phosphotriester or

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phosphate alkylate structure as well. In other
embodiments, the oligonucleotide analogs can have sugar
linking groups of the modified pyrimidines replaced with
carbon or ether linkages.

5   For therapeutics, it is convenient that the
oligonucleotide analog be prepared in a pharmaceutically
acceptable carrier.

             It is preferred in some embodiments of the
present invention to employ pyrimidine modified
10   oligonucleotides that are further modified. In this
context, pyrimidine modified oligonucleotide analogs refers
to structures that are generally similar to native
oligonucleotides, but which have been modified in one or
more significant ways.

15   Such modifications may take place at the sugar
backbone of the invention. It is generally preferred to
enhance the ability of the target sequence of the
pyrimidine modified oligonucleotides to penetrate into the
intracellular spaces of cells where the messenger RNA and
20   the DNA, which are the targets of the overall composition,
reside. Therefore, it is generally preferred to provide
modifications of oligo nucleotides that are substantially
less ionic than native forms in order to facilitate
penetration of the oligonucleotide into the intracellular
25   spaces. Any of the existing or yet to be discovered
methods for accomplishing this goal may be employed in
accordance with the practice of the present invention. At
present, it has been found preferable to employ
substitutions for the phosphodiester bond, which substi-
30   tutions are not only relatively less ionic than the
naturally occurring bonds but are also substantially
non-chiral. As will be appreciated, the phosphorus atom in
the phosphodiester linkage is "pro-chiral". Modifications
at the phosphorus, such as is done in methyl phosphonates
35   and phosphorothioates type oligonucleotides, results in
essentially chiral structures. Chirality results in the
existence of two isomers at each chiral center that may

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interact differently with cellular molecules. Such an
unresolved mixture of isomers may inhibit the transport of
the resulting compositions into the intracellular spaces or
decrease the affinity and specificity of hybridization to
5 the specific target RNA or DNA. Thus, it is preferred to
employ substantially non-ionic, substantially non-chiral
entities in lieu of some or all of the phosphodiester
bonds. For this purpose, short chain alkyl or cycloalkyl
structures especially C2-C4 structures are preferred. The
10 modification of the sugar structure including the
elimination of one of the oxygen functionality may permit
the introduction of such substantially non-chiral,
non-ionic substituents in this position.

In keeping with the goals of the invention, the
15 standard backbone modifications such as substituting S-P,
Me-P, MeO-P, or H₂N-P (that is phosphorothioates, methyl
phosphonates, phosphotriesters or phosphoamidates) for O-P
(phosphodiesters) are contemplated. These substitutions
are thought in some cases to enhance pyrimidine modified
20 oligonucleotide properties.

The targeting portion of the compositions of the
present invention, are preferably oligonucleotide analogs
having 5 to about 50 base units. It is more preferred that
such functionalities have from 8 to about 40 base units,
25 and even more preferred that from about 12 to 20 base units
be employed. Oligonucleotide analogs having about 15 base
units are preferable for the practice of certain
embodiments of the present invention.

It is desired that the targeting portion be
30 adapted so as to be specifically hybridizable with the
preselected nucleotide sequence of the RNA or DNA selected
for modulation. Oligonucleotide analogs particularly
suited for the practice of one or more embodiments of the
present invention comprise one or more subunits of modified
35 ribose or deoxyribose pyrimidines. The substitutions that
may occur at the 5 and 6 positions of the pyrimidine ring
may include additions of methyl, hydroxyl, alkoxy, alcohol,

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benzyl, or phenyl groups. The modifications may also
involve the addition of nitro groups, thiol groups, or
halogen groups. In the alternative, the modification may
be a carbon ring or heteroatom containing ring additions
5   fused to the 5 and 6 positions. The additions that occur
at the 5 and 6 positions may, but need not be, the same.

These modified bases are linked together and with
the rest of the oligonucleotide or oligonucleotide analog
through a sugar linking group. The linking group may be
10   any of those structures described herein that are capable
of linking sugar moieties of oligonucleotides together to
form the targeting portion of the compositions of this
invention. It is preferred that these sugar linking groups
comprise the phosphodiester structure or a derivative of
15   such. Derivatives of the phosphodiester structure may
include substitution of a sulphur, methyl, methyl oxide, or
amine group for an oxygen. The sugar phosphate nucleic
acid backbone may be modified as a phosphorothioate,
methylphosphonate, phosphoamidate or phosphotriester
20   moiety. The phosphodiester linkage may also be replaced by
a carbon or ether linkage.

In other embodiments of this invention, a linking
moiety has been devised to allow the direct attachment of a
modified pyrimidine to the terminal end of the 3'-end of
25   the modified oligonucleotides. Thus, an ester precursor
such as a bromomethylketo group, may be attached to the
3'-hydroxyl of a modified pyrimidine nucleoside that has
its 5'-hydroxyl protected with a dimethoxytrityl group (a
dimethoxytriphenylmethyl group) and, if necessary, its
30   pyrimidine heterocyclo protected with suitable protecting
group, as for instance a benzolate protecting group for the
cytosine series of nucleosides. If the required targeting
sequence has a terminal 3'-thymine or -cytosine base, the
desired modified thymine or cytosine base containing the
35   bromomethylketo linker can be utilized as the first monomer
to attach to the control pore glass (CPG) solid support
that contains a normal nucleoside attached via its

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3'-hydroxyl group. The base sensitive ester linkage attaching the modified pyrimidine to the nucleoside attached to the CPG may be cleaved under the usual concentrated ammonium hydroxide conditions that are
5   utilized to remove the oligonucleotide from the CPG support. This will allow the modified oligonucleotide to have a modified thymine or cytosine at it terminal 3'-end.

Cleavage of oligonucleotides by nucleolytic enzymes require the formation of an enzyme-substrate
10   complex, or in particular a nuclease-oligonucleotide complex. The nuclease enzymes will generally require specific binding sites located on the oligonucleotides for appropriate attachment. If the oligonucleotide binding sites are removed or hindered such that the nucleases will
15   not attach to the oligonucleotides, then nuclease resistent oligonucleotides result. In the case of restriction endonucleases that cleave sequence-specific palindromic double-stranded DNA, certain binding sites such as the ring nitrogen atoms in the 3- and 7-positions of purine bases
20   have been identified as required binding sites. Removal of one or more of these sites or hindering the nuclease approach to these particular positions within the recognition sequence has provided various levels of resistance to the specific nucleases.
25   A random structure-activity relationship approach was undertaken to discover nuclease resistant antisense oligonucleotides that maintained appropriate hybridization properties. A series of modifications to the 5- and/or 6-positions of the thymine and cytosine ribonucleosides and
30   deoxynucleosides were performed. These modified nucleosides were inserted into sequence-specific oligonucleotides via solid phase nucleic acid synthesis. The novel antisense oligonucleotides were assayed for their ability to resist degradation by nucleases and to possess
35   hybridization properties comparable to the unmodified parent oligo- nucleotide. Preferred embodiments where the 5- and 6- positions of the nucleic acid pyrimidine

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positions were selected because peripheral modifications
and ring modifications such as aza/deaza in these positions
are not likely to interfere sterically with required
Watson-Crick base pair hydrogen bonding patterns. However,
5   it is believed that profound electronic changes in an ring
system result from such seemingly inconsequential
heterocyclic changes. These electronic changes may add
dipole moments to the aromatic system and/or change
directions and strengths of existing dipole moments. Pka
10 strengths will be effected by the electronic perturbation.
Peripheral or ring changes as aza/deaza may change the
electrophilicy or nucleophilicy of the ring system such
that nucleases, which may require covalent bond formation
to a nucleic acid base of an oligonucleotide for their
15 degradative activity, will be inoperative.

During our structure activity relationship
studies, it was surprisingly discovered that peripheral and
aza/deaza modifications in the 5- and/or 6-positions of the
pyrimidines, thymine, cytosine, and uracil provided
20 resistance to nucleases which was comparable to
phosphorothioates, known for their nuclease resistance, yet
the hybridization properties of the modified
oligonucleotides were as good or better than the unmodified
parent oligonucleotides.

25   In some embodiments of this invention
modification of the pyrimidines nucleic bases will likely
allow about 50% of the antisense oligonucleotide to be
modified (two of the four bases available). Modified
sequences having all thymines and cytosines replaced with
30 6-aza-thymine and 6-aza-cytosine (8 out of 15 bases)
exhibited nuclease resistance with about a 10% lowering of
the T_m compared to the unmodified parent sequence. A
preferred combination of nuclease resistance and binding
stability results from placing one to three modified
35 pyrimidines at the 3'-end of the oligonucleotide.

The oligonucleotide analogs of this invention can
be used in diagnostics, therapeutics, and as research

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reagents and kits. For therapeutic use the oligonucleotide
analog is administered to an animal suffering from a
disease effected by some protein. It is preferred to
administer to patients suspected of suffering from such a
5   disease with amounts of oligonucleotide analog that are
effective to reduce the symptomology of that disease. It
is within the realm of a person's skill in the art to
determine optimum dosages and treatment schedules for such
treatment regimens.

10   It is generally preferred to apply the
therapeutic agents in accordance with this invention
internally such as orally, intravenously, or
intramuscularly. Other forms of administration, such as
transdermally, topically, or intra-lesionally may also be
15   useful. Inclusion in suppositories may also be useful.
Use of pharmacologically acceptable carriers is also
preferred for some embodiments.

EXAMPLES

EXAMPLE 1

20   Synthesis of 6-aza-5'-O-(dimethoxytriphenylmethyl)-
3'-O-(ß-cyanoethyldiisopropylphosphoramidityl)thymidine,
i.e.,
6-methyl-2-(5'-O-dimethoxytriphenylmethyl-3'-O-ß-cyano-
ethyldiisopropylphosphoramidityl-2'-deoxy-ß-D-erythro-
25   pentofuranosyl)-1,2,4-triazin-3,5(2H,4H)-dione.

A.   Synthesis of 6-Methyl-3.5-bis(trimethylsiloxy)-
1,2,4-triazine.

A mixture of 6-azathymine (purchased from Aldrich
Chemical Co.) (5.0 g, 39.4 mmol), hexamethyldisilazane
30   (HMDS) (15 ml), and chlorotrimethylsilane (TMSCl) (0.5 ml),
in a round bottom flask (50 ml) fitted with a condenser and
a drying tube, is refluxed by heating in an oil bath
(150°C); NH$_4$Cl collects as white powder in the condenser.
When a clear solution was obtained (~ 1 hr.), the excess of
35   HMDS/TMSCl was removed by distillation at 30°C/torr (bath
temp 100°C). The residual oil crystallized on drying under

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vacuum (0.1 torr), giving 6.57 g (61%) of 6-Methyl-3.5-
bis(trimethylsiloxy)-1,2,4-triazine, mp 43°C.

B.   Synthesis of 2-(2-Deoxy-3,5-di-0-p-toluoyl-β-D-
erythro-pentofuranosyl)-6-methyl-1,2,4-triazine-3,5(2H,4H)-
5   dione.
        A mixture of 6-Methyl-3,5-bis(trimethylsiloxy)-
1,2,4-triazine (4.0 g, 14.7 mmol) and 2-deoxy-3,5-di-0-p-
toluoyl-α-D-erythro-pentofuranosyl chloride (4.8 g, 12.4
mmol) in dry CHCl₃ (300ml) was stirred at room temperature
10 under anhydrous conditions. CuI (2.4 g, 12.4 mmol) was
added to the solution and the resulting slurry stirred for
3 hours at ambient temperature at which time thin layer
chromatography (TLC) indicated the reaction was complete.
The mixture was treated with saturated aqueous NaHCO₃ (200
15 ml), stirred for 15 minutes, and filtered through a pad of
celite that had been washed with CHCl₃ (2x50 ml). The
organic layer was washed with saturated NaCl (200 ml),
dried (MgSO₄) and concentrated to furnish a gummy residue.
This material was essentially one spot on TLC. A rapid
20 filtration through a short silica gel column using
EtOAc/hexane (1:1) provided several fractions of the
desired compounds. Crystallization of the residue from
EtOH furnished the product as white needles; 3.64 g (63%),
mp 170°C. A second crop was collected from the filtrate
25 (730 mg, 12.6%).

C.   Synthesis of 6-azathymidine [2-(2-deoxy-β-D-erythro-
pentofuranosyl)-6-methyl-1,2,4-triazine-3,5(2H,4H]-dione].
        To a mixture of the blocked nucleoside, 2-(2-Deoxy-
3,5-di-0-p-toluoyl-β-D-erythro-pentofuranosyl)-6-methyl-
30 1,2,4-triazine-3,5(2H,4H)-dione, described above (480 mg,1
mmol) in absolute MeOH (15 ml), was added dry NaOMe (50 mg)
while stirring at room temperature. TLC after one hour
indicated complete deblocking of the toluoyl groups. The
solution was rendered neutral by addition of one ml of
35 Dowex-50 (hydrogen form) ion-exchange resin. The

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suspension was filtered and the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in water (3 ml) and washed with CHCl$_3$ (3x5 ml). The aqueous layer was freeze-dried to provide a hard foam that was

5 further dried over P$_2$O$_5$ in a vacuum desiccator at 0.1 torr. The compound was obtained in 96% yield (230 mg) and was identical in all respects when compared to the literature values, e.g., UV, NMR TLC and mp.

D.   Synthesis of 2-[2-Deoxy-5'-(4,4'-dimethoxytrityl)-
10 β-D-erythro-pentofuranosyl]-6-methyl-1,2,4-triazine-
3,5(2H,4H)-dione. (6-azathymidine 5'-DMT)

        To a stirred solution of 6-azathymidine, synthesized as described above, (2.43 g, 10 mmol) in dry pyridine (100 ml) was added 4,4'-dimethoxytrityl chloride (4.06 g, 12
15 mmol) and 4-dimethylaminopyridine (120 mg, 1 mmol) at room temperature. The resulting yellow solution was stirred for 6 hours at room temperature, at which time TLC indicated a complete reaction. MeOH (10 ml) was added and the solution was concentrated under vacuum. The residue was dissolved
20 in CH$_2$Cl$_2$ (200 ml), extracted with saturated NaHCO$_3$ (100 ml) followed by sat. NaCl (100 ml), and then dried (MgSO$_4$). The filtrate and washings were combined and concentrated to provide a gummy residue that was purified by silica gel chromatography using EtOA$_c$/triethylamine (99/1) for elution.
25 Appropriate fractions were pooled and concentrated to furnish a white foam (3.82 g 70%); ^{1}H NMR (CDCl$_3$) δ 6.56 (t, 1H, C$_1$, H) and other protons.

E.   Synthesis of 2-[2'-Deoxy-5'-(4,4'-dimethoxytrityl)-
30 β-D-erythro-pentofuranosyl]-6-methyl-1,2,4-triazine-
3,5(2H,4H)-dione 3'-O-N,N-diisopropyl-β-cyanoethyl-
phosphoramidite.

        To a stirred solution of 6-azathymidine 5'-DMT, synthesized as described above, (1.63 g, 3 mmol) in dry THF
35 (50 ml) was added diisopropylethylamine (1.56 ml, 9 mmol) and solution was cooled to 0°C. N,N-Di-isopropyl-β-

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cyanoethylphosphonamidic chloride (1.42 ml, 6 mmol) was
added dropwise to the above solution over a period of 15
min. The reaction mixture was then stirred at room
temperature for 2 hours. Ethyl acetate (100 ml, containing
5 1% triethylamine) was added and the solution washed with
saturated NaCl solution (2 x 100 ml). The organic layer
was dried (MgSO$_4$) and the solvent removed under vacuum to
furnish a gummy residue. The product was purified by
silica gel column chromatography using EtOAc/triethylamine
10 (99/1, V/V) for elution. Pooling and concentration of
appropriate fractions furnished a white foam 1.41 g (66%);
^{1}H NMR (CDCl$_3$) δ 6.65 (m, 1, C$_1$.$\underline{H}$), 10.2 (brs, 1, N$\underline{H}$) and
other protons.

            Other species of 6-aza-pyrimidines may also be
15 synthesized using this procedure including 6-aza-2'-
deoxycytidine (6-Aza dC), 6-aza-2'-deoxyuridine (6-Aza dU),
6-aza-5-methylcytidine (6-Aza-5-Methyl C), 6-aza-5-
bromouridine (6-Aza-5-Bromo U), 5-fluorocytidine (5-Fluoro
C), and 5-bromocytidine (5-Bromo C). In addition, 5-aza-
20 uridine can be similarly synthesized from the starting
material 1,3,5-Triazin-2,4(1H,3H)-dione (Sigma Chemical).
EXAMPLE 2
            Procedure for the conversion of 5- and/or 6-modified
thymine and cytosine 2'-deoxyribonucleoside-5'-DMT-3'-
25 phosphoramidites into oligonucleotides.
            Table I depicts the protocol for the synthesis of
DNA on CPG supports. Prior to synthesis 5- and/or
6-modified thymine, cytosine 5'-dimethoxytriphenylmethyl
-2'-deoxyribonucleoside, or generally any nucleoside with
30 modifications in the heterocycle and/or sugar may be
attached to the 5'-hydroxyl of nucleosides bound to CPG
support in order to attach such a modified nucleoside at
the very 3' end of the oligonucleotide sequence.
            The modified thymidines or 2'-deoxycytidines that
35 will reside in the terminal 3'-position of certain
antisense oligonucleotides are protected by their 5'-DMT
(the cytosine 4-exocyclic amino is benzoylated) and

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treated with trifluoroacetic acid/bromoacetic acid mixed
anhydride in pyridine and dimethylaminopyridine at 50°C for
five hours. The solution is evaporated under reduced
pressure to a thin syrup that is dissolved in ethyl acetate
5   and passed through a column of silica gel. The homogenous
fractions were collected and evaporated to dryness. A
solution of 10 ml of acetonitrile, 10 micromoles of the
3'-O-bromomethylester modified pyrimidine nucleoside, and
one ml of pyridine/dimethylaminopyridine (1:1) is syringed
10   slowly (60 to 90 sec) through a one micromole column of CPG
thymidine (Applied Biosystems, Inc.) that had previously
been treated with acid according to standard conditions to
afford the free 5'-hydroxyl group. Other nucleoside bound
CPG columns could be employed. The eluent is collected and
15   syringed again through the column. This process is
repeated three times. The CPG column is washed slowly with
10 ml of acetonitrile and then attached to an ABI 380B
nucleic acid synthesizer. Oligonucleotide synthesis as
described in Table 1 is now initiated. The standard
20   conditions of concentrated ammonium hydroxide deprotection
that cleaves the thymidine ester linkage from the CPG
support also cleaves the 3',5' ester linkage connecting the
pyrimidine modified nucleoside to the thymidine that was
initially bound to the CPG nucleoside.
25   Standard synthesis of DNA may also be completed
according to Table I, on CPG LCAA using 1-10 micromoles of
nucleoside on the support. Polymer bound nucleosides were
purchased from Applied Biosystems, Inc. The synthesis was
accomplished using an automated synthesizer (ABI 380B) with
30   a continuous flow (3.0-3.5 ml/min). Phosphoramidite
condensations were completed under dry nitrogen. At the
completion of the synthesis, oligonucleotide cleavage and
deprotection were effected by treatment with concentrated
ammonia at 55°C for 24 hours. The purity and size of the
35   final products were confirmed by electrophoretic analysis
on polyacrylamide gels.

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TABLE I

Protocol for synthesis of DNA on CPG supports
(1-10 micromole scale)

Step	Reagent or Solvent Mixture	Time (min:sec)	Vol (ml)
1	Dichloroethane	1:30	5.0
2	3% DCA in dichloroethane	1:30	5.0
3	Dichloroethane	1:00	3.5
4	Acetonitrile	2:00	5.0
5	Condensation-Phosphoramidite In line mixing - 20 micromoles of phosphoramidite/CH_3CN and 3% Tetrazole in CH_3CN	1:00	3.5
	Recycling	1:00	---
6	Oxidation - 0.01 M Iodine in a mixture of $CH_3CN:H_2O:2$-lutidine	0:20	1.0
7	Capping (in line mixing)	1:00	3.5
	a) 20% $(AcO)_2$ in $CH_3CN:2$-lutidine (80:20) b) 3% DMAP in CH_3CN		
	Recycling	1:00	--

The synthesis cycles are combined into four basic
steps: 1) acidic treatment to remove 5'-dimethoxytrityl
protecting groups; 2) condensation of the polymer bound
nucleoside with a nucleoside-3'-diisopropylphosphoramidite
for DNA and 3) oxidation, using iodine and water in
acetonitrile, to convert the phosphite linkage into a
phosphate linkage; and 4) capping with acetic anhydride and
DMAP to block off unreacted sites and to remove residual
moisture.

     After assembly of the desired sequence by the
automated synthesizer, the final product was cleaved from
the polymer, deprotected and isolated.

     The deprotection was accomplished by treatment of
the polymer support with ammonium hydroxide (NH_4OH) at 55°C

for 20 hours. The crude material was purified by trityl-
on HPLC. Detritylation was accomplished with 3%
ethylacetate followed by extractions with ethylacetate.
Purification was accomplished by sodium chloride/ethanol
5 precipitation (2X) and HPLC. All of the above mixed
sequences were characterized by kinasing the samples and
sizing them.

EXAMPLE 3

       Synthesis of the 6-aza-thymine-modified oligo-
10 nucleotides, listed below, was accomplished according to
Example 2 wherein the procedure for the attachment of 5-
and/or 6- modified thymine or cytosine 5'-dimethoxytri-
phenylmethyl-2'-deoxyribonucleoside to the 5' hydroxyl of
nucleosides bound to a CPG support was provided. The
15 phosphoramidites synthesized according to the method
provided in Example 1 are incorporated into
oligonucleotides having the following sequences that are
useful due to their nuclease resistant characteristics.

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OLIGONUCLEOTIDES HAVING 6-AZA-THYMIDINE (T') SUBSTITUTIONS

SEQUENCE	SUBSTITUTION POSITION(S)
T'T'T'T'T'T'T'T'T'T'T'T'T'T'	1 - 14
AT'AT'AT'AT'AT'AT'AT'AT'	2,4,6,8,10,12,14
T'CCAGGT'GT'CCGCAT [1]	1,7,9
T'CCAGGT'GT'CCGCAT'C	1,7,9,15
T'CCAGGT'GT'CCGCAT'C	1,7,9
T'CCAGGTGTCCGCAT'C	1,15
TCCAGGTGTCCGCAT'C	15
T'CCAGGTGTCCGCATC	1
T'CCAGGT'GTCCGCATC	1,7
TCCAGGT'GT'CCGCATC	7,9
T'CCAGGT'GT'CCGT'T'T'C	1,7,9,13,14,15
T'CCAGGT'GT'CCGT'sT'sT'sC [2]	1,7,9,13,14,15
CGACT'AT'GCAAGT'AC [3]	5,7,13
CGACTATGCAAT'T'T'C [3]	12,13,14
T'T'T'CTATGCAAGTAC [3]	1,2,3
GT'CCAT'GT'CGT'ACGC [4]	2,6,8,11
ACCGAGGT'CCAT'GT'CGT'ACGC [4]	8,12,14,17
AC'C'GAGGT'C'C'AT'GT'C'GT'AC'GC [4,5]	8,12,14,17
CCT'T'CCCCT'C [6]	3,4,9

[1]   selected sequence from 5 lipoxygenase genome

[2]   P of the phosphodiester bond is replaced by an S at
       the 13,14, and 15 positions

[3]   selected sequence from the papilloma virus genomes

[4]   selected sequence from the herpes simplex virus
       genomes

[5]   6 aza-cytidine substitutions at the 2,3,9,10,15 and
       19 positions

[6]   sequence found especially effective as a triplex

EXAMPLE 4

      Synthesis of 6-aza-cytosine modified
oligonucleotides, listed below, was accomplished according
to Example 2 wherein a method for the conversion of 5-
and/or 6- modified thymine and cytosine 2'

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deoxyribonucleoside-5-DMT-3'-phosphoramidites into
oligonucleotides is provided. The phosphoramidites
synthesized according to the method specified in Example 1
are substituted for wild type bases of the oligonucleotides
5 of the following sequences in order to increase nuclease
resistance.

OLIGONUCLEOTIDES HAVING 6-AZA-CYTIDINE SUBSTITUTIONS

SEQUENCE	SUBSTITUTION POSITION(S)
10 C'C'C'C'C'C'C'C'C'C'C'C'C'C'C	1 - 14
C'C'C'AGGTGTCCGCATC [1]	1,2,3
TCCAGGTGTCCGCATC	10,11
TCCAGGTGTCCGC'C'C'C	13,14,15
TCCAGGTGTCCGCC'C'C	14,15
15 CGACTATGCAAC'C'C'C [2]	12,13,14
C'C'C'CTATGCAAGTAC	1,2,3
C'GAC'TATGC'AAGTAC	1,4,9
AC'C'GAGGTC'C'ATGTC'GTAC'GC	2,3,9,10,15,19
AC'C'GAGGT'C'C'AT'GT'C'GT'AC'GC [3]	2,3,9,10,15,19

20   [1]   sequence selected from 5 lipoxygenase genome

          [2]   sequence selected from papilloma virus genomes

          [3]   6 aza-thymidine substitutions at positions
                  8,12,14 and 17

EXAMPLE 5

25   Conversion of 5- and/or 6-position modified thymines
to the corresponding thymidines (deoxyribosylation).
          The thymine analogs are trimethylsilylated under
various standard conditions such as hexamethyldisilazane
(HMDS) and an acid catalyst (i.e., ammonium chloride) and
30 then treated with 3,5-O-ditoluoyl-2-deoxy-α-D-erythro-
pentofuranosyl chloride in the presence a variety of Lewis
acid catalysts (i.e., stannic chloride, iodine, boron
tetrafluoroborate, etc.). A specific procedure has
recently been described by J. N. Freskos, *Nucleosides &*

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Nucleotides, 8:1075-1076 (1989), in which copper (I) iodide
is the catalyst employed.

EXAMPLE 6

Synthesis of 6-aza-5-bromo-5'-O-(dimethoxytriphenyl-
5   methyl)-3'-O-(ß-cyanoethyldiisopropylphosphoramidityl)-
2'-deoxyuridine, i.e., 6-bromo-2-(5'-O-[dimethoxytriphenyl-
methyl]-3-O-[ß-cyanoethyldiisopropylphosphoramidityl]-
2'-deoxy-ß-<u>D</u>-<u>erythro</u>-pentofuranosyl)-1,2,4-triazin-3,5-
(2H,4H)-dione.

10   The required heterocycle, 6-Bromo-1,2,3-triazin-
3,5(2H,4H)-dione, was prepared according C. Cristescu, *Rev.
Roumaine Chim.* 20:1287 (1975). Deoxyribosylation of this
material and subsequent DMT and amidite chemistry is
performed according to the procedures set forth in

15 Examples 1, 2 and 5.

EXAMPLE 7

Conversion of 5- and/or 6-position modified
thymidines to the corresponding 2'deoxycytidines (chemical
conversion of an pyrimidine type 4-keto group to an 4-amino

20 group).

The 3',5'-sugar hydroxyls of the modified thymidine
types are protected by acyl groups such as toluoyl,
benzoyl, p-nitrobenzoyl, acetyl, isobutyl, trifluoroacetyl,
etc. using standards conditions of the acid chlorides or

25 anhydrides and pyridine/dimethylaminopyridine solvent and
catalysts. The protected nucleoside is chlorinated with
thionyl chloride or phosphoryl chloride in pyridine or
other appropriate basic solvents. The pyrimidine type
4-chloro groups are next displaced with ammonium in

30 methanol. Deprotection of the sugar hydroxyls also takes
place. The amino group is benzoylated by the standard
two-step process of complete benzylation (sugar hydroxyls
and amino group) and the acyls are selectively removed by
aqueous sodium hydroxide solution. Alternatively, the in

35 situ process of first treating the nucleoside with
chlorotrimethylsilane and base to protect the sugar
hydroxyls from subsequent acylation may be employed.

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Another conversion approach is to replace the pyrimidine
type 4-chloro group with an 1,2,4-triazolo group that
remains intact throughout the oligonucleotide synthesis on
the DNA synthesizer and is displaced by ammonium during the
5   ammonium hydroxide step that removes the oligonucleotide
from the CPG support and deprotection of the heterocycles.
Furthermore, in many cases the pyrimidine type 4-chloro
group can be utilized as just described and replaced at the
end of the oligonucleotide synthesis. 5-aza-cytidine and
10   5-nitro-cytidine may accordingly be obtained from its
uridine analog. Likewise, 4-chloro-5-methylmercapto-
cytidine is synthesized as described.

EXAMPLE 8

Synthesis of 6-aza-5-bromo-5'-O-(dimethoxytriphenyl-
15   methyl)-3'-O-(ß-cyanoethyldiisopropylphosphoramidityl)-
2'-deoxycytidine, i.e.,
5-amino-6-bromo-2-(5'-O-dimethoxytriphenylmethyl-3'-O-ß-cya
noethyldiisopropylphosphoramidityl-2'-deoxy-ß-D-<u>erythro</u>-
pentofuranosyl)-1,2,4-triazin-3(2H)-one.
20   This monomer is prepared by the conversion of the
deoxyribosylated 6-Bromo-1,2,3-triazin-3,5(2H,4H)-dione
(according to Example 7) to the 2'-deoxycytidine analog
according to Example 5.

EXAMPLE 9

25   1.   Synthesis of 5-Trifluoro-5'-O-(dimethoxytri-
phenylmethyl)-3'-O-(ß-cyanoethyldiisopropylphosphor-
amidityl)-2'-deoxyuridine, i.e., 1-(5'-[4,4'-
dimethoxytrityl]-2'-deoxy-β-<u>D</u>-<u>erythro</u>-pentofuranosyl)5,5,5-
trifluorothymine.
30   Trifluorothymidine (1, 2.0 g), (purchased from PCR,
Inc., Gainsville, FL), was twice coevaporated with pyridine
and then dissolved in anhydrous pyridine (100 ml) under an
argon atmosphere. 4,4'-Dimethylaminopyridine (50 mg) and
4,4'-dimethoxytrityl chloride (2.74 g) were successively
35   added to the solution which was then stirred at room
temperature for 6 hr. The reaction mixture was treated
with 100 ml of water then repeatedly extracted with diethyl

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ether (4 x 100 ml). The ether extracts were pooled, dried
over magnesium sulfate, filtered and then evaporated in
vacuo to afford an orange gum. This gum was coevaporated
with toluene (3 x 25) ml) and then chromatographed on 120 g
5   silica gel using ethyl acetate/hexanes/triethylamine
(49/49/2, v/v) as eluent. The appropriate fractions were
pooled and evaporated to yield 0.77 g (19%) of a solid
foam. 'H-NMR (DMSO-d6) δ 8.05 (s, 1H, H-6); 7.4-6.7 (2m,
13H, aromatic); 6.05 (t, 1H, H-1').
10   2.   Synthesis of 1-(5'-[4,4'-Dimethoxytrityl]-2'-
deoxy-β-<u>D</u>-<u>erythro</u>-pentofuranosyl)-5,5,5-trifluorothymine-
3'-<u>O</u>-N,N-diisopropyl-β-cyanoethyl phosphoramidite (3).
        To a stirred solution of 5-Trifluoro-5'-O-
(dimethoxytriphenylmethyl)-3'-O-(β-cyanoethyldiisopropyl-
15 phosphoramidityl)-2'-deoxyuridine, i.e., 1-(5'-[4,4'-
dimethoxytrityl]-2'-deoxy-β-<u>D</u>-<u>erythro</u>-pentofuranosyl)
5,5,5-trifluorothymine (2) (0.70 g, 1.2 mMol) in anhydrous
THF was added diisopropylethyl amine (1.0ml) under an argon
atmosphere. To this solution was added N,N-diisopropyl-
20 cyanoethylphosphoramidic chloride (0.33ml, 1.44 mMol)
dropwise over a period of 5 min. The mixture was stirred
at room temperature for 3 hr. At the end of this time, the
reaction mixture was evaporated to dryness <u>in vacuo</u> and the
gummy residue that resulted was dissolved in CH_2Cl_2 (75 ml).
25 The solution was washed with satd. $NaHCO_3$ (2 X 35 ml) and
brine (35ml). The organic layer was separated, dried over
magnesium sulfate, filtered and evaporated in vacuo to
afford a colorless gum. This material was chromatographed
on 60 g of silica gel using $CHCl_3$/triethylamine (99/1, v/v)
30 as eluent. The appropriate fractions were pooled and
evaporated to afford a colorless foam (0.6 g, 67%). 'H-NMR
(DMSO-d6): δ 8.15 (s, 1H, H-6); 7.4-6.8 (1m, 13H,
aromatic); 6.05 (t, 1H, H-1'). This phosphoramidite may be
subsequently incorporated into oligonucleotides according
35 to the procedure set forth in Example 2. Some examples
follow.

OLIGONUCLEOTIDES HAVING 5-TRIFLUOROMETHYL-2'-DEOXYURIDINE SUBSTITUTIONS

SEQUENCE	SUBSTITUTION POSITION(S)
T'CCAGGTGTCCGCATC [1]	1
T'CCAGGT'GTCCGCATC	1,7
T'CCAGGT'GT'CCGCATC	1,7,9
TCCAGGT'GT'CCGCATC	7,9
TCCAGGTGTCCGCAT'C	15
CGACTATGCAAT'T'T'C [2]	12,13,14
T'T'T'CTATGCAAGTAC	1,2,3
ACCGAGGT'CCAT'GT'CGT'ACGC [3]	8,12,14,17

[1]   sequence selected from 5 lipoxygenase genome

[2]   sequence selected from papilloma virus genomes

[3]   sequence selected from herpes simplex virus genomes

EXAMPLE 10

    5-Fluoro-5'-O-(dimethoxytriphenylmethyl)-3'-O-(ß-cyanoethyldiisopropylphosphoramidityl)-2'-deoxyuridine was purchased from Glen Research Corporation. This has previously been inserted into oligonucleotides. J. F. Habener, et al., *Proceedings of the National Academy of Sciences of the U.S.A.*, 85:1735-1739 (1988). Some examples are listed below.

OLIGONUCLEOTIDES HAVING 5-FLUORO-2'-DEOXYURIDINE SUBSTITUTIONS

SEQUENCE	SUBSTITUTION POSITION(S)
T'T'T'T'T'T'T'T'T'T'T'T'T'T'T	1 - 15
TCCAGGTGTCCGCAT'C [1]	15
TCCAGGT'T'CCGCAT'C	7,9
T'CCAGGT'GT'CCGCAT'C	1
T'CCAGGTGTCCGT'T'T'C	13,14,15
T'CCAGGT'GT'CCGCAT'C	1,7,9,15

[1]   sequence selected from 5 lipoxygenase genome

EXAMPLE 11

5-Bromo-5'-O-(dimethoxytriphenylmethyl)-3'-O-(ß-cyanoethyldiisopropylphosphoramidityl)-2'-deoxyuridine, purchased from Glen Research Corporation, was incorporated into oligonucleotide sequences during synthesis according to the procedure set forth in Example 2.

OLIGONUCLEOTIDES WITH 5-BROMO-2'-DEOXYURIDINE SUBSTITUTIONS

SEQUENCE	SUBSTITUTION POSITION(S)
T'T'T'T'T'T'T'T'T'T'T'T'T'T'T	1 - 15
TCCAGGTGTCCGCAT'C [1]	15
T'CCAGGTGTCCGCATC	1
TCCAGGT'GT'CCGCATC	7,9
TCCAGGTGTCCGT'T'T'C	13,14,15
T'CCAGGT'GT'CCGCAT'C	1,7,9,15

[1]   sequence selected from 5 lipoxygenase genome

EXAMPLE 12

Synthesis of 5-bromo-2'-deoxycytidine-modified oligonucleotides, listed below, was accomplished according to the procedure set forth in Example 2.

OLIGONUCLEOTIDES HAVING 5-BROMO-2'-DEOXYCYTIDINE (C')
SUBSTITUTIONS

SEQUENCE	SUBSTITUTED POSITION(S)
C'C'C'C'C'C'C'C'C'C'C'C'C'C'C	1 - 15
C'CCAGGTGTCCGCAT'C [1]	1
TCCAGGTGTCCGCAC'C	15
TCCAGGTGTC'C'GCATC	10,11
TCCAGGTGTCCGC'C'C'C	13,14,15
TC'C'AGGTGTC'C'GC'ATC	2,3,10,11,13

[1]   sequence selected from 5 lipoxygenase genome

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EXAMPLE 13

    Synthesis of 5-methyl-2'-deoxycytidine-modified oligonucleotides, listed below, was accomplished according to the procedure set forth in Example 2 by substituting 5
5 methyl-2'deoxycytidine (Glen Research Corporation) for wild type cytidine bases.

OLIGONUCLEOTIDES HAVING 5-METHYL-2'-DEOXYCYTIDINE (C') SUBSTITUTIONS

SEQUENCE	SUBSTITUTED POSITIONS
C'C'C'C'C'C'C'C'C'C'C'C'C'C'C	1 - 15
C'CCAGGTGTCCGCAT'C [1]	1
TCCAGGTGTCCGCAC'C	15
TCCAGGTGTC'C'GCATC	10,11
TCCAGGTGTCCGC'C'C'C	13,14,15
TC'C'AGGTGTC'C'GC'ATC	2,3,10,11,13

    [1]   sequence selected from 5 lipoxygenase genome

EXAMPLE 14

    Synthesis of 5-iodo-2'-deoxyuridine-modified oligonucleotides, listed below, was accomplished according
20 to the procedure set forth in Example 2 by substituting 5-iodo-2'-deoxyuridine (Glen Research Corporation) for wild type uridine bases.

OLIGONUCLEOTIDES HAVING 5-IODO-2'-DEOXYURIDINE SUBSTITUTIONS

SEQUENCE	SUBSTITUTED POSITION(S)
T'T'T'T'T'T'T'T'T'T'T'T'T'T'T	1 - 15
TCCAGGTGTCCGCAT'C [1]	15
T'CCAGGTGTCCGCATC	1
TCCAGGT'GT'CCGCATC	7,9
TCCAGGTGTCCGT'T'T'C	13,14,15
T'CCAGGT'GT'CCGCAT'C	1,7,9,15

    [1]   sequence selected from 5 lipoxygenase genome

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EXAMPLE 15

The synthesis of 5-iodo-5'-O-(dimethoxytriphenyl-
methyl)-3'-O-(ß-cyanoethyldiisopropylphosphoramidityl)-2'-
deoxycytidine is accomplished by the utilization of the
5   procedures set forth in Examples 2, and 7.

EXAMPLE 16

Synthesis of 5-chloro-5'-O-(dimethoxytriphenyl-
methyl)-3'-O-(ß-cyanoethyldiisopropylphosphoramidityl)-2'-
deoxyuridine.

10   5-Chlorouracil is commercially available from
Aldrich Chemical Company. This material is converted to
the title phosphoramidite according to the procedures set
forth in Examples 1, 2 and 5.

EXAMPLE 17

15   The synthesis of 5-chloro-5'-O-(dimethoxytriphenyl
methyl)-3'-O-(ß-cyanoethyldiisopropylphosphoramidityl)-2'-
deoxycytidine is accomplished according to the procedures
set forth in Examples 2 and 7.

EXAMPLE 18

20   Synthesis of 5-nitro-5'-O-(dimethoxytriphenyl-
methyl)-3'-O-(ß-cyanoethyldiisopropylphosphoramidityl)-2'-
deoxyuridine.

5-Nitrouracil is commercially available from Aldrich
Chemical Company. This material is converted to the title
25   phosphoramidite according to Examples 1, 2 and 5.

EXAMPLE 19

Synthesis of 5-(2,4-dinitrophenylmercapto-5'-O-
(dimethoxytriphenylmethyl)-3'-O-(ß-cyanoethyldiisopropyl-
aminophosphinyl)-2'-deoxyuridine.

30   The 5-mercapto-2'-deoxyuridine, obtained from the
procedure of Fox et al., *Journal of the American Chemical
Society* 81:178 (1959), is converted to the 2,4-dinitro-
phenylsulfinyl derivative by treatment with an equivalent
of sodium hydride and 2,4-dinitrophenylfluoride. This
35   material is converted to the 5'-DMT-3'-phosphoramidite as
described in procedures set forth in Example 1.

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EXAMPLE 20

Synthesis of 4-chloro-5-(2,4-dinitrophenylmercapto)-
5'-O-(dimethoxytriphenylmethyl)-3'-O-(b-cyanoethyldiiso-
propylaminophosphinyl)-2'-deoxycytidine.

The 5-mercapto-2'-deoxyuridine, obtained from the
procedure of Fox et al., *Journal of the American Chemical
Society* 81:178 (1959), is converted to the 2,4-dinitro-
phenylsulfinyl derivative by treatment with an equivalent
of sodium hydride and 2,4-dinitrophenylfluoride. This
material is acetylated, chlorinated, and converted to the
5'-DMT-3'-phosphoramidite as described in Example 1 and 5.

EXAMPLE 21

Synthesis of 5-methylmercapto-5'-O-(dimethoxytri-
phenylmethyl)-3'-O-(ß-cyanoethyldiisopropylaminophos-
phinyl)-2'-deoxyuridine.

The 5-mercapto-2'-deoxyuridine, obtained from the
procedure of Fox et al., *Journal of the American Chemical
Society* 81:178 (1959), is converted to the methyl
derivative by treatment with an equivalent of sodium
hydride and methyliodide. This material is converted to
the 5'-DMT-3'-phosphoramidite as described in procedures
set forth in Example 1.

EXAMPLE 22

The ability of pyrimidine modified oligonucleotides
to hybridize to their complementary RNA or DNA sequences
was determined by thermal melting analysis. The RNA
complement was synthesized from T7 RNA polymerase and a
template-promoter of DNA synthesized with an Applied
Biosystems, Inc. 380B synthesizer. The RNA species was
purified by ion exchange using FPLC (LKB Pharmacia,Inc.).
Either natural antisense oligonucleotides or those
containing pyrimidine modifications at specific locations
were added to either the RNA or DNA complement at
stoichiometric concentrations and the absorbance (260 nm)
hyperchromicity upon duplex to random coil transition was
monitored using a Gilford Response II spectrophotometer.
These measurements were performed in a buffer of 10 mM

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Na-phosphate, pH 7.4, 0.1 mM EDTA, and NaCl to yield an
ionic strength of 10 either 0.1 M or 1.0 M. Data was
analyzed by a graphic representation of 1/Tm vs ln[Ct],
where [Ct] was the total oligonucleotide concentration.
From this analysis the thermodynamic parameters were
determined. The results of certain of these tests are
shown in Table II.

TABLE II

MELTING TEMPERATURE (Tm) DATA ON MODIFIED
OLIGONUCLEOSIDES5' TCC AGG TGT CGG CAT C 3'position 1 to 16
reading from 5' to 3'ModifiedSites(s) ofTm
(°C)PyrimidineModification6-Aza T162.81562.07, 958.21, 7,
9, 1554.56-Aza dC263.02, 359.810, 1151.06-Me
dU163.21562.57, 955.71, 7, 9, 1552.35,6-DiMe dU1560.37,
954.71, 7, 9, 1552.55-Iodo dU163.51563.37, 963.31, 7, 9,
1561.55-Bromo dU163.51563.37, 963.31, 7, 9, 1561.55-Fluoro
dU163.21563.27, 965.01, 7, 9, 1563.75-Bromo dC10, 1165.82,
3, 10, 11, 1368.05-Me dC10, 1165.32, 3, 10, 11, 1367.0

Based upon the information gained concerning the
stability of the duplex of heteroduplex formed, the
placement of modified pyrimidine into oligonucleotides were
assessed for their effects on helix stability.
Modifications that drastically alter the stability of the
hybrid exhibit reductions in the free energy (delta G) and
decisions concerning their usefulness as antisense
oligonucleotides were made. Representative studies of such
stabilities are shown in Table III.

TABLE III

EFFECTS OF PYRIMIDINE MODIFICATIONS ON DUPLEX STABILITYAND
SPECIFITYCompoundStability vsSpecificity vsDNA TargetDNA
Targetnatural DNA0+4.26-Aza T+0.6+3.76-Aza dC+1.9+6.06-Me
dU+1.3+3.05,6-DiMe dU+1.1+1.55-Iodo dU+0.3-5-Bromo dU-
0.3+4.95-Fluoro dU-0.2+4.45-Bromo dC-0.1+6.55-Methyl dC-
0.2+6.3

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The ability of the pyrimidine modified antisense
oligonucleotides to hybridize with specificity to the
targeted mRNA was shown by Northern blot analysis of
purified target mRNA in the presence of total cellular RNA.
5   Target mRNA was synthesized from a vector containing the
cDNA for the target mRNA located downstream from a T7 RNA
polymerase promoter. Synthesized mRNA was electrophoresed
in an agarose gel and transferred to a suitable support
membrane (i.e., nitrocellulose). The support membrane was
10 blocked and probed using [^{32}P]-labeled antisense
oligonucleotides.

The stringency was determined by replicate blots and
washing at either elevated temperatures or decreased ionic
strength of the wash buffer. Autoradiography was performed
15 to assess the presence of heteroduplex formation and the
autoradiogram quantitated by laser densitometer (LKB
Pharmacia, Inc.).

The specificity of hybrid formation was determined
by isolation of total cellular RNA by standard techniques
20 and its analysis by agarose electrophoresis, membrane
transfer and probing with the labeled pyrimidine modified
oligonucleotides. Stringency was predetermined for the
unmodified antisense oligonucleotides and the conditions
used such that only the specifically targeted mRNA was
25 capable of forming a heteroduplex with the pyrimidine
modified oligonucleotide.

EXAMPLE 23

Natural, phosphorothioate, and pyrimidine modified
oligonucleotides were assessed for their resistance to
30 serum nucleases by incubation of the oligonucleotides in
media containing various concentrations of fetal calf serum
(FCS) or adult human serum. Labeled oligonucleotides were
incubated for various times, treated with protease K and
then analyzed by gel electrophoresis on 20%
35 polyacrylamide-urea denaturing gels and subsequent
autoradiography. Autoradiograms were quantitated by laser
densitometry. Based upon the location of the modifications

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and the known length of the oligonucleotide, it was
possible to determine the effect on nuclease degradation by
the particular pyrimidine modification. For the
cytoplasmic nucleases, a HL60 cell line was used. A
5   post-mitochondrial supernatant was prepared by differential
centrifugation and the labeled oligonucleotides were
incubated in this supernatant for various times. Following
the incubation, oligonucleotides were assessed for
degradation as outlined above for serum nucleolytic
10   degradation. Autoradiography results were quantitated for
comparison of the unmodified, the phosphorothioates, and
the pyrimidine modified oligonucleotides.

       Evaluation of the resistance of natural and
pyrimidine-modified oligonucleotides to specific nucleases
15   (i.e., endonucleases, 3',5'-exo-, and 5',3'-exonucleases)
was done to determine the exact effect of the modifications
on degradation. Modified oligonucleotides were incubated
in defined reaction buffers specific for various selected
nucleases. Following treatment of the products with
20   proteinase K, urea was added and analysis on 20%
polyacrylamide gels containing urea was done. Gel products
were visualized by staining using Stains All (Sigma
Chemical Co.). Laser densitometry was used to quantitate
the extent of degradation. The effects of the pyrimidine
25   modifications were determined for specific nucleases and
compared with the results obtained from the serum and
cytoplasmic systems.

       In one such test about 1mg/ml of modified pyrimidine
bearing oligonucleotides were incubated at 37°C in DMEM
30   supplemented with 10% heat activated FCS. At various time
points aliquots were removed and mixed in an equal volume
of 9M urea/1x TBE buffer and frozen at -20°C. The samples
were analyzed by polyacrylamide electrophoresis (20% PA/7M
urea) and visualized by Stains All reagent and subjected
35   to quantitation using an LKB laser densitometer.
Integrations of peaks representing the full length
oligonucleotide and the n-1 oligonucleotide were made. The

% degradation was calculated for each time point by
comparison to the time point 0 of sample. The data was
graphed and graphic estimations for $T_{1/2}$ of the modified
oligonucleotide was made by comparison to the natural

5   unmodified oligonucleotide. Utilizing 6-aza T containing
oligonucleotide it was found that such 6-aza T modified
oligonucleotide exhibited greater stability towards
nucleolytic degradation. For the 6-aza T compounds,
increasing the number of modification increased the

10   stability towards degradation in FCS. Furthermore, the
best results were obtained when 6-aza T was used as a 3´-
terminus cap. An oligonucleotide containing such a 3'-
terminus cap provided protection from 3´-5´ exonucleolytic
degradation compared to a natural oligonucleotide. T1/2

15   increased from 20 min. for a natural oligonucleotide to a
T1/2 of 4 h for the modified oligonucleotide. This
represented a 12-fold decrease in the susceptibility of
oligonucleotide to degradation in presence of FCS. The
results of this test suggests that FCS, which is known to

20   hydrolyse natural unmodified oligonucleotides from the
3´end, does not recognize a block of three 6-aza T as an
appropriate substrate. Such lack of recognition decreases
the hydrolysis of modified oligonucleotide considerably.
EXAMPLE 24

25   Ribonuclease H activity
        Cleavage of the target mRNA by RNase H greatly
enhances the inhibitory effects of antisense
oligonucleotides. It is presently believed that the E.
Coli RNase H requires only 3 to 4 unmodified residue

30   binding site on the DNA portion of the DNA-RNA heteroduplex
to elicit cleavage of the RNA. For this test an antisense
oligonucleotide to 5LO mRNA (5´-AAA TAG TGT TGC TGA TCT TGA
C-3´) having 6-aza T substituted for each T nucleotide was
utilized for RNase H testing. In the test oligonucleotide

35   the positions modified to contain 6-aza T were each
separated by no more than two nature bases - thus the
oligonucleotide should demonstrate recognition by E. Coli

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RNase H. A natural complementary DNA (5´AAA TAG TGT TGC
TGA TCT TGA C-3´) was synthesized for comparison.
The 5-LO mRNA (2.5kb) was synthesized in vitro. The
natural DNA (AAA TAG TGT TGC TGA TCT TGA C)

5   and the 6-aza T containing modified oligonucleotide (AAA
TAG TGT TGC TGA TCT TGA C), both being antisense DNA to 5-
LO mRNA, were synthesized by automated DNA synthesis. The
mRNA was heated for 30 min at 60°C in the presence of a 3
times molar excess of each of the natural and modified

10 oligonucleotide, and with the natural oligonucleotide as a
control. Following incubation, the solutions were slowly
cooled to 37°C and RNase H (E. Coli) was added. The RNase
H treated solutions were allowed to stand at 37°C for 30
min. The degradation products were analyzed by 1.2%

15 agarose/formaldehyde electrophoresis and visualized with
ethidium bromide for quantitation. The results indicated
that RNase H (E. Coli) was able to recognize and cleave
mRNA bound to either natural or modified DNA containing 6-
aza T with equal effectiveness.

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What is claimed is:

1. An oligonucleotide analog having at least one pyrimidine base modified to have the structure:
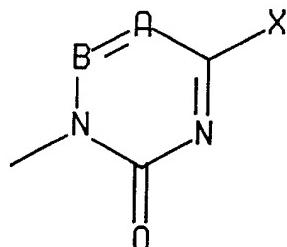
wherein X= OH or NH_2; and

5   A and B may be the same or different and may be: C-lower alkyl, N, $C-CF_3$, C-F, $C-C(CF_3)$, $C-CF_2-CF_3$, $C-CF_2-CF_2-CF_3$, C-Cl, C-Br, C-I, C-halocarbon, $C-NO_2$, $C-OCF_3$, C-SH, $C-SCH_3$, C-OH, C-O-lower alkyl, $C-CH_2OH$, $C-CH_2SH$, $C-CH_2SCH_3$, $C-CH_2OCH_3$, $C-NH_2$, $C-CH_2NH_2$, $C-alkyl-NH_2$, C-benzyl, C-aryl, C-

10   aralkyl, C-substituted aryl, C-substituted benzyl; or one of A and B is as above and the other is C-H; or together A and B are part of a carbocyclic or heterocyclic ring fused to the pyrimidine ring through A and B.

2. The oligonucleotide analog of claim 1

15   wherein A is C-lower alkyl, C-O-lower alkyl, C-OH, C-phenyl, C-benzyl, C-nitro, C-thiol, C-halocarbon, or C-halogen.

3. The oligonucleotide analog of claim 1 wherein B is C-lower alkyl, C-O-lower alkyl, C-OH, C-

20   phenyl, C-benzyl, C-nitro, C-thiol, C-halocarbon, or C-halogen.

4. The oligonucleotide analog of claim 1 wherein A and B are each, independently, C-lower alkyl, C-O-lower alkyl, C-OH, C-phenyl, C-benzyl, C-nitro, C-thiol,

25   C-halocarbon, or C-halogen.

5. The oligonucleotide analog of claim 1 wherein at least one of A and B is C-halogen or C-halocarbon.

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6.   The oligonucleotide analog of claim 1 wherein at least one of A and B is nitrogen.

7.   The oligonucleotide analog of claim 1 wherein at least one of A and B is C-CH$_3$ or C-CF$_3$.

8.   The oligonucleotide analog of claim 1 wherein A is C-CH$_3$ or C-CF$_3$ and B is nitrogen.

9.   The oligonucleotide analog of claim 1 wherein A is C-Br and B is nitrogen.

10   The oligonucleotide analog of claim 1 wherein said modified pyrimidine is at one end of said oligonucleotide analog.

11.   The oligonucleotide analog of claim 1 wherein said modified pyrimidine is at the 3' end of said oligonucleotide analog.

12.   The oligonucleotide analog of claim 1 wherein up to about 3 modified pyrimidines are incorporated at the 3' end of said oligonucleotide.

13.   The oligonucleotide analog of claim 1 wherein at least about 1% of said pyrimidines are modified.

14.   The oligonucleotide analog of claim 1 wherein at least about 10% of said pyrimidines are modified.

15.   The oligonucleotide of claim 1 wherein at least about 25% of said pyrimidines are modified.

16.   The oligonucleotide of claim 1 wherein at least about 50% of said pyrimidines are modified.

17.   The oligonucleotide of claim 1 wherein substantially all of said pyrimidines are modified.

18.   The oligonucleotide analog of claim 1 wherein the sugar moiety of the pyrimidine is ribose or deoxyribose.

19.   The oligonucleotide analog of claim 1 wherein the sugar linking groups of the modified pyrimidine are modified to a phosphorothioate, methyl phosphonate, or phosphate alkylate.

20.   The oligonucleotide analog of claim 1
wherein the sugar linking groups of the modified pyrimidine
are replaced with a carbon or ether linkage.

21.   The oligonucleotide analog of claim 1 in a
5 pharmaceutically acceptable carrier.

22.   The oligonucleotide analog of claim 1 which
exhibits improved nuclease resistance as compared to
corresponding wild type oligonucleotides.

23.   A method for modulating the production of a
protein by an organism comprising contacting the organism
with an oligonucleotide analog specifically hybridizable
with a selected nucleic acid sequence coding for said
5 protein, wherein at least one pyrimidine base of said
oligonucleotide analog is modified to have the structure:
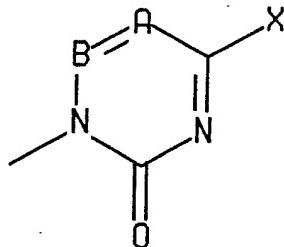
wherein X= OH or NH_2; and
    A and B may be the same or different and may be:
C-lower alkyl, N, $C-CF_3$, C-F, $C-C(CF_3)$, $C-CF_2-CF_3$, $C-CF_2-$
10 CF_2-CF_3, C-Cl, C-Br, C-I, C-halocarbon, $C-NO_2$, $C-OCF_3$, C-SH,
$C-SCH_3$, C-OH, C-O-lower alkyl, $C-CH_2OH$, $C-CH_2SH$, $C-CH_2SCH_3$,
$C-CH_2OCH_3$, $C-NH_2$, $C-CH_2NH_2$, alkyl-NH_2, C-benzyl, C-aryl, C-
aralkyl, C-substituted aryl, C-substituted benzyl; or one
of A and B is as above and the other is C-H; or together A
15 and B are part of a carbocyclic or heterocyclic ring fused
to the pyrimidine ring through A and B.

24.   The method of claim 23 wherein A is C-lower
alkyl, C-O-lower alkyl, C-OH, C-phenyl, C-benzyl, C-nitro,
C-thiol, C-halocarbon, or C-halogen.

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20   25. The method of claim 23 wherein B is C-lower
alkyl, C-O-lower alkyl, C-OH, C-phenyl, C-benzyl, C-nitro,
C-thiol, C-halocarbon, or C-halogen.

            26. The method of claim 23 wherein A and B are
each, independently, C-lower alkyl, C-O-lower alkyl, C-OH,
25 C-phenyl, C-benzyl, C-nitro, C-thiol, C-halocarbon, or C-
halogen.

            27. The method of claim 23 wherein at least one
of A and B is C-halogen or C-halocarbon.

            28. The method of claim 23 wherein at least one
30 of A and B is nitrogen.

            29. The method of claim 23 wherein at least one
of A and B is $C-CH_3$ or $C-CF_3$.

            30. The method of claim 23 wherein A is $C-CH_3$ or
$C-CF_3$ and B is nitrogen.

35   31. The method of claim 23 wherein A is C-Br and
B is nitrogen.

            32. The method of claim 23 wherein said modified
pyrimidine is at one end of said oligonucleotide analog.

            33. The method of claim 23 wherein said modified
40 pyrimidine is at the 3' end of said oligonucleotide analog.

            34. The method of claim 23 wherein up to about 3
modified pyrimidines are incorporated at the 3' end of said
oligonucleotide.

            35. The method of claim 23 wherein at least
45 about 1% of said pyrimidines are modified.

            36. The method of claim 23 wherein at least
about 10% of said pyrimidines are modified.

            37. The method of claim 23 wherein at least
about 25% of said pyrimidines are modified.

50   38. The method of claim 23 wherein at least
about 50% of said pyrimidines are modified.

            39. The method of claim 23 wherein substantially
all of said pyrimidines are modified.

            40. The method of claim 23 wherein the sugar
55 moiety of the pyrimidine is ribose or deoxyribose.

41.   The method of claim 23 wherein the sugar linking groups of the modified pyrimidine are modified to a phosphorothioate, methyl phosphonate, or phosphate alkylate.

42.   The method of claim 23 wherein the sugar linking groups of the modified pyrimidine are replaced with a carbon or ether linkage.

43.   The method of claim 23 wherein said oligonucleotide analog is in a pharmaceutically acceptable carrier.

44.   The method of claim 23 wherein said oligonucleotide analog exhibits improved nuclease resistance as compared to corresponding wild type oligonucleotides.

45.   A method for treating an organism having a disease characterized by the undesired production of a protein comprising contacting the organism with an oligonucleotide analog hybridizable with a selected nucleic acid sequence coding for said protein, either alone or in a pharmaceutically acceptable carrier, wherein at least one pyrimidine base of said oligonucleotide analog is modified to have the structure:
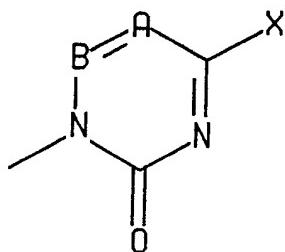
wherein   X= OH or NH_2; and

A and B may be the same or different and may be: C-lower alkyl, N, $C-CF_3$, C-F, $C-C(CF_3)$, $C-CF_2-CF_3$, $C-CF_2-CF_2-CF_3$, C-Cl, C-Br, C-I, C-halocarbon, $C-NO_2$, $C-OCF_3$, C-SH, $C-SCH_3$, C-OH, C-O-lower alkyl, $C-CH_2OH$, $C-CH_2SH$, $C-CH_2SCH_3$, $C-CH_2OCH_3$, $C-NH_2$, $C-CH_2NH_2$, $alkyl-NH_2$, C-benzyl, C-aryl, C-aralkyl, C-substituted aryl, C-substituted benzyl; or one of A and B is as above and the other is C-H; or together A

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and B are part of a carbocyclic or heterocyclic ring fused to the pyrimidine ring through A and B.

　　　46.　The method of claim 45 wherein A is C-lower alkyl, C-O-lower alkyl, C-OH, C-phenyl, C-benzyl, C-nitro, C-thiol, C-halocarbon, or C-halogen.

　　　47.　The method of claim 45 wherein B is C-lower alkyl, C-O-lower alkyl, C-OH, C-phenyl, C-benzyl, C-nitro, C-thiol, C-halocarbon, or C-halogen.

　　　48.　The method of claim 45 wherein A and B are each, independently, C-lower alkyl, C-O-lower alkyl, C-OH, C-phenyl, C-benzyl, C-nitro, C-thiol, C-halocarbon, or C-halogen.

　　　49.　The method of claim 45 wherein at least one of A and B is C-halogen or C-halocarbon.

　　　50.　The method of claim 45 wherein at least one of A and B is nitrogen.

　　　51.　The method of claim 45 wherein at least one of A and B is $C-CH_3$ or $C-CF_3$.

　　　52.　The method of claim 45 wherein A is $C-CH_3$ or $C-CF_3$ and B is nitrogen.

　　　53.　The method of claim 45 wherein A is C-Br and B is nitrogen.

　　　54.　The method of claim 45 wherein said modified pyrimidine is at one end of said oligonucleotide analog.

　　　55.　The method of claim 45 wherein said modified pyrimidine is at the 3' end of said oligonucleotide analog.

　　　56.　The method of claim 45 wherein up to about 3 modified pyrimidines are incorporated at the 3' end of said oligonucleotide.

　　　57.　The method of claim 45 wherein at least about 1% of said pyrimidines are modified.

　　　58.　The method of claim 45 wherein at least about 10% of said pyrimidines are modified.

　　　59.　The method of claim 45 wherein at least about 25% of said pyrimidines are modified.

　　　60.　The method of claim 45 wherein at least about 50% of said pyrimidines are modified.

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61. The method of claim 45 wherein substantially
all of said pyrimidines are modified.

62. The method of claim 45 wherein the sugar
moiety of the pyrimidine is ribose or deoxyribose.

63. The method of claim 45 wherein the sugar
linking groups of the modified pyrimidine are modified to a
phosphorothioate, methyl phosphonate, or phosphate
alkylate.

64. The method of claim 45 wherein the sugar
linking groups of the modified pyrimidine are replaced with
a carbon or ether linkage.

65. The method of claim 45 wherein said
oligonucleotide analog is in a pharmaceutically acceptable
carrier.

66. The method of claim 45 wherein said
oligonucleotide analog exhibits improved nuclease
resistance as compared to corresponding wild type
oligonucleotides.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/04681

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5); A61K 48/00; C07H 21/00
U.S.Cl: 514/44, 536/27, 28, 29

II FIELDS SEARCHED

Minimum Documentation Searched 7	
Classification System	Classification Symbols
U.S.Cl:	514/44; 536/27, 28, 29

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 8

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
Y,P	US, A, 4,948,882 (Ruth) 14 August 1990, See column 4, lines 44-58; column 7, lines 6-62.	1-23
Y	US, A, 4,711,955 (WARD et al) 08 December 1987, see entire document.	1-23
Y	Anti-Cancer Drug Design, Volume 2 issued 1987, P.S. Miller et al., "A New Approach to Chemotherapy based on molecular Biology and Nucleic Acid Chemistry Matagen (masking tape for gene expression)", pages 117-128, see entire document.	23-66

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10 December 1991	**1 0 JAN 1992**
International Searching Authority	Signature of Authorized Officer
ISA/US	James O. Wilson

Form PCT/ISA/210 (second sheet) (Rev.11-87)